PCT/AU99/00343

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WITNESS my hand this Nineteenth day of May 1999

KIM MARSHALL
MANAGER EXAMINATION SUPPORT AND SALES

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AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: "NOVEL ANTI-FIBRINOLYTIC AGENTS"

The invention is described in the following statement:

TITLE

"NOVEL ANTI-FIBRINOLYTIC AGENTS"

FIELD OF THE INVENTION

THIS INVENTION relates to anti-fibrinolytic agents and in particular, novel plasmin inhibitors having reduced propensity for causation of rebound thrombosis. The present invention also relates to amino acid sequences and nucleotide sequences encoding the novel plasmin inhibitors as well as to methods of producing these inhibitors and pharmaceutical compositions containing same.

BACKGROUND OF THE INVENTION

The blood loss associated with major forms of surgery has in the past been compensated by replacement therapy which may involve fresh frozen plasma, fresh whole blood and platelet concentrates. With recent awareness of a variety of blood borne viral infections (Hepatitis B and C, and human immunodeficiency virus, HIV), the need to reduce blood loss during surgery is a major priority. Further anxiety has been generated within National Blood Transfusion Services concerning infectivity with agents related to Bovine Spongiform Encephalitis (BSE) and Creuzfeldt-Jacob's Disease (CJD) for which there is no reliable assay at the present time.

It has been established (Royston, 1990, *Blood Coagul. Fibrinol.* **1**:53-69; Orchard *et al*, 1993, *Br. J. Haemat.* **85**:596-599) that unfettered fibrinolytic activity via the plasminogen-plasmin pathway

contributes to hemorrhage and that a plasmin inhibitor such as aprotinin helps alleviate blood loss. This seems to suggest that plasmin-mediated digestion of fibrin clots and components of the coagulation system may be of primary importance as a contribution to this hemorrhagic state (Orchard *et al*, 1993, *supra*).

The use of aprotinin during cardiopulmonary bypass (CPB) surgery is now commonplace (Royston, 1990, *supra*; Orchard *et al,* 1993, *supra*). In particular, Orchard *et al (*1993, *supra*) have demonstrated that the bovine source inhibitor aprotinin, as the active substance in the medicament Trasylol™, reduces blood loss in CPB patients by neutralization of plasmin activity and does not affect platelet activity. This latter finding has been confirmed by other investigators (Ray and March, 1997, *Thromb. Haemost.* **78**:1021-1026).

Aprotinin is a well investigated serine protease inhibitor, or *'serpin'*. It comprises 58 amino acids and acts to inhibit trypsin, α-chymotrypsin, plasmin as well as tissue and plasma kallikrein (Fritz and Wunderer, 1983, *Drug Res.* **33**:479-494; Gebhard *et al*, 1986 *In "Proteinase Inhibitors"*, Barrett and Salvesen (eds.), Elsevier Science Publications BV pp 374-387). Aprotinin has also been found to react with thrombin and the plasminogen activators (tPA and uPA) (Willmott *et al*, 1995, *Fibrinolysis* **9**:1-8).

Recent studies have shown that semi-synthetically generated homologs of aprotinin which contain other amino acids in place

of lysine at position 15 of the amino acid sequence have a profile of action and specificity of action which differ distinctively from those of aprotinin (US Patent No 4,595,674; Wenzel *et al*, 1985, *In "Chemistry of Peptides and Proteins"* Vol. 3). Some of these semi-synthetic aprotinin homologs have, for example, a strongly inhibiting action on elastase from pancreas and leucocytes. Other aprotinin homologs with arginine at position 15, alanine at position 17, and serine at position 42, are characterized by an inhibitory action which is distinctly greater than that of aprotinin on plasma kallikrein (*cf.* WO 89/10374).

Reference also may be made to US Patent No 5,576,294 (Norris *et al*) which discloses human protease inhibitors of the same type as aprotinin. In particular, there is disclosed variants of human Kunitz-type protease inhibitor that preferentially inhibit neutrophil elastase, cathepsin G and/or proteinase 3. Compared to aprotinin, these variants have a net negative charge and are considered to have a reduced risk of kidney damage when administered to patients in large doses. In contrast, aprotinin has a nephrotoxic effect when administered in relatively high doses (Bayer, *Trasylol, Inhibitor of proteinase*; Glaser *et al*, *In "Verhandlungen der Deutchen Gesellschaft Für Innere Medizin, 78. Kongress"*, Bergmann, München, 1972, pp 1612-1614). This nephrotoxicity is considered to be a consequence of the strongly net positive charge of aprotinin which causes it to bind to the negatively charged surfaces of kidney tubuli.

While there is no doubt that the anti-fibrinolytic clinical use of aprotinin reduces blood loss during vascular surgery, there is evidence of increased incidence of *'rebound thrombosis'* which manifests in graft occlusion and perioperative myocardial infarction (Van der Meer *et al,* 1996, *Thromb. Haemost.* **75**:1-3; Cosgrove *et al*, 1992, *Annals Thorac. Surg.* **54**:1031-1038; Samama *et al*, 1994, *Thromb. Haemost.* **71**:663-669). Consistent with these findings, it has been shown that aprotinin has a somewhat broad specificity and slow tight-binding kinetic action on plasmin (Willmott *et al*, 1995, *supra*). Accordingly, the increased incidence of rebound thrombosis may be a consequence of the tight binding of aprotinin to plasmin and concomitant irreversible neutralization of the fibrinolytic system.

Until recently, there were no effective anti-fibrinolytic agents described in the prior art with reduced propensity for causation of rebound thrombosis compared to aprotinin. However, in a recent study, Willmott *et al* (1995, *supra*) isolated and characterized a plasmin inhibitor from the venom of the Australian brown snake, *Pseudonaja textilis textilis* with a promising kinetic profile in respect of rebound thrombosis. This isolated preparation of plasmin inhibitor, termed Textilinin (Txln), was found to consist of a single aproximately 7 kDa protein, as assessed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. In contrast to the many serine protease enzymes inhibited by aprotinin, Txln was only shown to inhibit plasmin

and trypsin. It was also shown to conform to a single stage competitive reversible mechanism for the binding of plasmin as opposed to a two stage reversible mechanism of aprotinin in which enzyme and virgin inhibitor react to initially produce a loose non-covalent complex followed by a tightly bound, stable complex in which enzyme and inhibitor remain largely unchanged (Laskowski and Kato, 1980, *Annu. Rev. Biochem.* **49**:593-626; Travis and Salvesen, 1983, *Annu. Rev. Biochem.* **52**:655-709; Longstaff and Gaffney, 1991, *Biochemistry* **30**:979-986). Moreover, TxIn was shown to bind plasmin more rapidly (dissociation rate constant, $k_{-1}=3.85x10^{-5}$ sec^{-1} M^{-1}) and with a less avid K_i (dissociation constant, K_i $=1.4x10^{-8}$ M) than aprotinin (dissociation rate constant, k_{-2} = $1.64x10^{-5}$ sec^{-1} M^{-1}; dissociation constant, $K_{i'}$ = $5.3x10^{-11}$ M - this latter value being in close agreement with a previously reported value of $K_{i'}$ = $2x10^{-10}$ M (Longstaff and Gaffney, 1992, *Fibrinolysis* **3**:89-87)). It was suggested therefore that the TxIn kinetic profile may be clinically more attractive with respect to rebound thrombosis than that of aprotinin in the management of perioperative and postoperative bleeding.

DISCLOSURE OF THE INVENTION

The present invention results from the unexpected discovery of two different plasmin inhibitors in the plasmin inhibitor preparation of Willmott *et al* (1995, *supra*) which was considered initially to be substantially homogeneous. Surprisingly, these plasmin inhibitors, termed Textilinin 1 (TxIn 1) and Textilinin 2 (TxIn 2) co-migrate with a molecular

mass of about 7 kDa, as assessed by SDS-PAGE, and constitute only about 50% of the total protein (by weight) in the parent plasmin inhibitor preparation used by Willmott and colleagues. This, together with the fact that TxIn 1 and TxIn 2 each have a different kinetic profile compared to the parent preparation, suggests that the parent preparation contains other compounds which may interfere with plasmin inhibition. In particular, TxIn 1 and TxIn 2 have distinct amino acid sequences, somewhat similar kinetic profiles (TxIn 1, $k_{-1}=3.09\times10^{-6}$ sec^{-1} M^{-1}; $K_i=3.5\times10^{-9}$ M; TxIn 2, $k_{-1} = 8.20\times10^{-6} sec^{-1} M^{-1}$; $K_i=2.0\times10^{-9}$ M), while both inhibit blood loss in a murine model. Like the parent counterpart, TxIn 1 and TxIn 2 react only with plasmin and trypsin and therefore have high enzyme specificity compared to aprotinin. Moreover, comparison of the respective kinetic profiles of TxIn 1, TxIn 2 and aprotinin for plasmin reveals that TxIn 1 and TxIn 2 are between 10-fold and 100-fold less efficient than aprotinin in inhibiting plasmin, and dissociate from plasmin between 10-fold and 100-fold more rapidly than aprotinin. Due to their high specificity for plasmin and low inhibitory efficiency, TxIn 1 and TxIn 2 may therefore have a therapeutic advantage, compared to aprotinin, to transiently affect the delicate balance between enzymes and inhibitors of the fibrinolytic system controlling the fluidity of blood.

It is therefore an object of the present invention to provide novel anti-fibrinolytic agents with reduced propensity for causation of rebound thrombosis.

Thus, in one aspect of the invention, there is provided a substantially pure preparation of a plasmin inhibitor characterized in that it is a single stage competitive inhibitor of plasmin.

The term "substantially pure" as used herein describes a compound, *e.g.*, a peptide which has been separated from components which naturally accompany it. Typically, a compound is substantially pure when at least 60%, more preferably at least 75%, more preferably at least 90%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole percent or mole fraction) in a sample is the compound of interest. Purity can be measured by any appropriate method, *e.g.*, in the case of peptides by chromatography, gel electrophoresis or HPLC analysis. A compound, *e.g.*, a peptide is also substantially purified when it is essentially free of naturally associated components when it is separated from the native contaminants which accompany it in its natural state.

Preferably, said single-stage competitive inhibitor has a dissociation constant for plasmin in the range of from $1x10^{-8}$ M^{-1} to $1x10^{-10}$ M^{-1}, more preferably from $5x10^{-8}$ M^{-1} to $8x10^{-9}$ M^{-1}, most preferably from $1x10^{-9}$ M^{-1} to $5x10^{-9}$ M^{-1}.

The single-stage competitive inhibitor may have a dissociation rate constant for plasmin in the range of from $4x10^{-5}$ sec^{-1} M^{-1} to $5x10^{-7}$ sec^{-1} M^{-1} , more preferably from $1x10^{-6}$ sec^{-1} M^{-1} to $1x10^{-7}$ sec^{-1} M^{-1}, most preferably from $2x10^{-6}$ sec^{-1} M^{-1} to $9x10^{-6}$ sec^{-1} M^{-1}.

Preferably, the single-stage competitive inhibitor comprises a peptide. To this extent, the single-stage competitive inhibitor may comprise the sequence of amino acids: Met-Lys-Asp-Arg-Pro-Asp-Phe-Cys-Glu-Leu-Pro-Ala-Asp-Thr-Gly-Pro-Cys-Arg-Val-Arg-Phe-Pro-Ser-Phe-Tyr-Tyr-Asn-Pro-Asp-Glu-Lys-Lys-Cys-Leu-Glu-Phe-Ile-Tyr-Gly-Gly-Cys-Glu-Gly-Asn-Ala-Asn-Asn-Phe-Ile-Thr-Lys-Glu-Glu-Cys-Glu-Ser-Thr-Cys-Gly-Ser [SEQ ID NO:1] or a biologically active fragment thereof. Alternatively, the single stage competitive inhibitor may comprise the sequence of amino acids: Met-Lys-Asp-Arg-Pro-Glu-Leu-Cys-Glu-Leu-Pro-Pro-Asp-Thr-Gly-Pro-Cys-Arg-Val-Arg-Phe-Pro-Ser-Phe-Tyr-Tyr-Asn-Pro-Asp-Glu-Gln-Lys-Cys-Leu-Glu-Phe-Ile-Tyr-Gly-Gly-Cys-Glu-Glu-Asn-Ala-Asn-Ala-Phe-Ile-Thr-Lys-Glu-Glu-Cys-Glu-Ser-Thr-Cys-Gly-Gly [SEQ ID NO:2] or a biologically active fragment thereof.

For the purposes of the present invention, the term "biologically active fragment" means a fragment of a substantially full-length parent peptide wherein the fragment retains the single stage competitive inhibition properties of the parent peptide with respect to plasmin.

The invention also contemplates peptides which are deemed to have at least 70% homology, more preferably at least 80% homology and most preferably at least 90% homology to the amino acid sequences defined by SEQ ID NO:1 and SEQ ID NO:2. Accordingly, the invention encompasses 'peptide homologs' of the peptides according to SEQ ID

NO:1 and SEQ ID NO:2. Thus, the invention also includes within its scope peptides which are functionally similar to those defined in SEQ ID NO:1 and SEQ ID NO:2. For example, one of skill in the art will appreciate that conservative amino acid substitutions can be made in peptides according to SEQ ID NO:1 and SEQ ID NO:2 (parent peptides) and that such substituted peptides will retain the functional characteristics of the parent peptides. For example, conservative amino acid substitutions typically include substitutions within the following groups:

glycine, alanine;

valine, isoleucine, leucine;

aspartic acid, glutamic acid;

asparagine, glutamine;

serine, threonine;

lysine, arginine; and

phenylalanine, tyrosine.

The present invention also encompasses functional derivatives of the peptides or peptide homologs of the invention. Such derivatives may include amino acid deletions and/or additions to peptides according to SEQ ID NO:1 and SEQ ID NO:2 or homologs thereof wherein said derivatives retain single stage competitive inhibition against plasmin. "Additions" of amino acids may include fusion of the peptide or peptide homologs with other peptides, polypeptides or proteins. It will be appreciated in this regard that the peptides or peptide homologs of the

invention may be incorporated into larger peptides, and such larger peptides may also be expected to retain single stage competitive inhibition against plasmin.

Other derivatives contemplated by the invention include analogues of the peptides or peptide homologs which include , but are not limited to, modification to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the peptide or peptide homologs of the invention.

For example, modifications contemplated to produce the analogues of the invention may include *in vivo* or *in vitro* chemical derivatization of peptides according to SEQ ID NO:1 or SEQ ID NO:2 or peptide homologs thereof, *e.g.*, amidation, acetylation, or carboxylation, and modifications of glycosylation, *e.g.*, those made by modifying the glycosylation patterns of a peptide or polypeptide during its synthesis and processing or in further processing steps, *e.g.*, by exposing the peptide to enzymes which affect glycosylation, *e.g.*, mammalian glycosylating or deglycosylating enzymes. Also embraced are peptides or peptide homologs of the invention which have phosphorylated amino acid residues, *e.g.*, phosphotyrosine, phosphoserine, or phosphothreonine.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by

acylation with acetic anhydride; acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; amidination with methylacetimidate; carbamoylation of amino groups with cyanate; pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$; reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; and trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS).

The carboxyl group may be modified by carbodiimide activation via O-acylisourea formation followed by subsequent derivitization, by way of example, to a corresponding amide.

The guanidine group of arginine residues may be modified by formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

Sulphydryl groups may be modified by methods such as performic acid oxidation to cysteic acid; formation of mercurial derivatives using 4-chloromercuriphenylsulphonic acid, 4-chloromercuribenzoate;2-chloromercuri-4-nitrophenol, phenylmercury chloride, and other mercurials; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; carboxymethylation with iodoacetic acid or iodoacetamide; and carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified, for example, by alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or

sulphonyl halides or by oxidation with N-bromosuccinimide.

Tyrosine residues, may be modified by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

The imidazole ring of a histidine residue may be modified by N-carbethoxylation with diethylpyrocarbonate or by alkylation with iodoacetic acid derivatives.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include but are not limited to, use of 4-amino butyric acid, 6-aminohexanoic acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, t-butylglycine, norleucine, norvaline, phenylglycine, ornithine, sarcosine, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acids contemplated by the present invention is shown in TABLE 1.

The invention also contemplates the use of crosslinkers, for example, to stabilise 3D conformations of the peptides or peptide homologs of the invention, using homo-bifunctional cross linkers such as bifunctional imido esters having $(CH_2)_n$ spacer groups with n = 1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety or carbodiimide. In addition, peptides can be conformationally constrained, for example, by introduction of double bonds between C_α and C_β atoms of amino acids, by incorporation of C_α

and N_α-methylamino acids, and by formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini between two side chains or between a side chain and the N or C terminus of the peptides or analogues. For example, reference may be made to: Marlowe (1993, *Biorganic & Medicinal Chemistry Letters* **3**:437-44, hereby incorporated by reference) which describes peptide cyclization on TFA resin using trimethylsilyl (TMSE) ester as an orthogonal protecting group; Pallin and Tam (1995, *J. Chem. Soc. Chem. Comm.* 2021-2022, hereby incorporated by reference) which describes the cyclization of unprotected peptides in aqueous solution by oxime formation; Algin *et al* (1994, *Tetrahedron Letters* **35**: 9633-9636, hereby incorporated by reference) which discloses solid-phase synthesis of head-to-tail cyclic peptides *via* lysine side-chain anchoring; Kates *et al* (1993, *Tetrahedron Letters* **34**: 1549-1552, hereby incorporated by reference) which describes the production of head-to-tail cyclic peptides by three-dimensional solid phase strategy; Tumelty *et al* (1994, *J. Chem. Soc. Chem. Comm.* 1067-1068, hereby incorporated by reference) which describes the synthesis of cyclic peptides from an immobilized activated intermediate, wherein activation of the immobilized peptide is carried out with *N*-protecting group intact and subsequent removal leading to cyclization; McMurray *et al* (1994, *Peptide Research* **7**:195-206, hereby incorporated by reference) which discloses head-to-tail cyclization of peptides attached to insoluble supports by means of the

side chains of aspartic and glutamic acid; Hruby *et al* (1994, *Reactive Polymers* **22**:231-241, hereby incorporated by reference) which teaches an alternate method for cyclizing peptides *via* solid supports; and Schmidt and Langer (1997, *J. Peptide Res.* **49**:67-73, hereby incorporated by reference) which discloses a method for synthesizing cyclotetrapeptides and cyclopentapeptides. The foregoing methods may be used to produce conformationally constrained peptides which single stage competitive inhibition kinetics in respect of plasmin.

The invention also contemplates peptides according to SEQ ID NO:1 or SEQ ID NO:2 or peptide homologs respectively thereof which have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a therapeutic agent.

The present invention further encompasses chemical analogues of peptides according to SEQ ID NO:1 or SEQ ID NO:2 or peptide homologs respectively thereof which analogues act as functional analogues of said peptides or peptide homologs. In this regard, chemical analogues may not necessarily be derived from said peptides or peptide homologs but may share certain conformational similarities. Alternatively, chemical analogues may be specifically designed to mimic certain physical properties of said peptides or peptide homologs. Chemical analogues may be chemically synthesized or may be detected following,

for example, natural product screening.

The peptides of the invention may be prepared using any suitable procedure. Preferably, such peptides are synthesized either manually or by using an automated peptide synthesizer.

Peptides according to SEQ ID NO:1 and SEQ ID NO:2 and peptide homologs thereof may be synthesized using solution synthesis or solid phase synthesis as described, for example, in Chapter 9 entitled *"Peptide Synthesis"* by Atherton and Shephard which is included in a publication entitled *"Synthetic Vaccines"* edited by Nicholson and published by Blackwell Scientific Publications.

Alternatively, a peptide or peptide homolog in accordance with the invention may be prepared by a procedure including the steps of:

(a) ligating a nucleotide sequence encoding a peptide according to SEQ ID NO:1, SEQ ID NO:2 or peptide homolog thereof into a suitable expression vector to form an expression construct;

(b) transforming or transfecting a suitable host cell with the expression construct;

(c) expressing the recombinant peptide; and

(d) isolating the recombinant peptide.

As used in this specification, an expression construct is a nucleotide sequence comprising a first nucleotide sequence encoding a peptide according to SEQ ID NO:1, SEQ ID NO:2 or peptide homolog thereof wherein said first sequence is operably linked to regulatory

nucleotide sequences (such as a promoter and a termination sequence) that will facilitate expression of said first sequence. Both constitutive and inducible promoters may be useful adjuncts for expression of the peptides according to the invention. The expression construct preferably includes a vector, such as a plasmid cloning vector. A vector according the invention may be a prokaryotic or a eukaryotic expression vector, which are well known to those of skill in the art.

Suitable host cells for expression may be prokaryotic or eukaryotic. Examples of prokaryotic host cells include bacteria. Examples of eukaryotic host cells include yeast as well as insect cells such as, for example, *Spodoptera frugiperda* 9 (Sf-9) cells.

Preferably, the expression vector is a baculovirus expression vector and the host cell is a Sf-9 insect cell.

The recombinant peptide may be conveniently prepared by a person skilled in the art using standard protocols as for example described in Sambrook *et al.* (1989, second edition, Cold Spring Harbor Laboratory Press, in particular Sections 16 and 17).

The term "nucleotide sequence" as used herein designates mRNA, RNA, cRNA, cDNA or DNA.

A nucleotide sequence encoding the peptides or peptide homologs of the invention may be conveniently prepared by taking advantage of the genetic code and synthesizing, for example, by use of an oligonucleotide sequencer, a sequence of nucleotides which when

translated by a host cell results in the production of a peptide according to SEQ ID NO:1, SEQ ID NO:2 or peptide homolog thereof. Alternatively, the nucleotide sequence may comprise a portion of a *txln 1* or a *txln 2* nucleic acid molecule (see hereinafter, FIGS. 6 and 7, respectively) coding for SEQ ID NO:1 or SEQ ID NO:2, respectively.

Accordingly, in another aspect, the invention resides in an isolated nucleic acid encoding the peptide according to SEQ ID NO:1, said nucleic acid comprising the sequence of nucleotides: ATGAAGGACCGGCCTGATTTTTGTGAACTGCCTGCTGACACCGGAC CATGTAGAGTCAGATTCCCATCCTTGTACTACAACCCAGATGAAAAAA AATGCCTCGAGTTTATTTATGGTGGATGCGAAGGGAATGCTAACGAT TTTATGACCAAAGAGGAGTGTGAAAGCACGTGTGG(N)AGT [SEQ ID NO:3].

In yet another aspect, the invention provides an isolated nucleic acid encoding the peptide according to SEQ ID NO:2, said nucleic acid comprising the sequence of nucleotides: ATGAAGGACCG GCCTGAGTTGTGTGAACTGCCTCCTGACACCGGACCATGTAGAGTCA GATTCCCATCCTTGTACTACAACCCAGATGAACAAAAATGCTGTAGA GTCAGATTCCCATCCTTGTACTACAACCCAGATGAACAAAAATGCAAA GAGGAGTGTGAAAGCACGTGTCC(N)GGT [SEQ ID NO:4].

An "isolated nucleic acid", as used herein, refers to a nucleic acid sequence, segment, or fragment which has been purified from the sequences which flank it in a naturally occurring state, *e.g.*, a DNA

fragment which has been removed from the sequences which are normally adjacent to the fragment, *e.g.*, the sequences adjacent to the fragment in a genome in which it naturally occurs. The term applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid, *e.g.*, RNA, or DNA or proteins which naturally accompany it in the cell.

The invention also contemplates homologs of the above 'wild-type' nucleotide sequences. Such "nucleotide sequence homologs" may comprise nucleotide sequences having at least 70% homology, more preferably at least 80% homology, and most preferably at least 90% homology to the nucleotide sequences defined by SEQ ID NO:3 and SEQ ID NO:4. Alternatively, the nucleotide sequence homologs may comprise nucleotide sequences which hybridize with a wild-type nucleotide sequence according to the invention under substantially stringent conditions. Suitable hybridization conditions will be discussed hereinafter.

The nucleotide sequence homologs of the invention may be prepared according to the following procedure:

(i) obtaining a nucleic acid extract from a suitable host;

(ii) creating primers which are preferably degenerate wherein each comprises a portion of a wild-type nucleotide sequence of the invention; and

(iii) using said primers to amplify, via nucleotide sequence amplification techniques, one or more nucleic acid

fragments from said nucleic acid extract.

Suitable nucleotide sequence amplification techniques are well known to the skilled addressee, and include polymerase chain reaction (PCR), strand displacement amplification (SDA) and rolling circle replication (RCR).

As used herein, a "nucleic acid fragment" refers to a nucleic acid product generated by nucleotide sequence amplification techniques.

The suitable host from which a nucleic acid extract is obtained is preferably a snake. Suitable snakes may be selected from the group consisting of the family *Elapidae*, and the family *Viperae*. By "obtained from" is meant that the nucleic acid extract is isolated from, or derived from, a particular source of the host. For example, the nucleic acid extract may be obtained from tissue isolated directly from the host.

Alternatively, a nucleotide sequence homolog of the invention may be obtained from a nucleotide sequence library derived from a tissue of a snake. Such a library may be a snake cDNA library or snake genomic DNA library.

"Hybridization" is used here to denote the pairing of complementary bases of distinct nucleotide sequences to produce a DNA-DNA hybrid, a DNA-RNA hybrid, or an RNA-RNA hybrid according to base-pairing rules.

In DNA, complementary bases are:

(i) A and T; and

(ii) C and G.

In RNA, complementary bases are:

(i) A and U; and

(ii) C and G.

In RNA-DNA hybrids, complementary bases are:

(i) A and U;

(ii) A and T; and

(iii) G and C.

Typically, substantially complementary nucleotide sequences are identified by blotting techniques that include a step whereby nucleotides are immobilized on a matrix (preferably a synthetic membrane such as nitrocellulose), a hybridization step, and a detection step. Southern blotting is used to identify a complementary DNA sequence; northern blotting is used to identify a complementary RNA sequence. Dot blotting and slot blotting can be used to identify complementary DNA/DNA, DNA/RNA or RNA/RNA polynucleotide sequences. Such techniques are well known by those skilled in the art, and have been described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Eds. Ausubel et al; John Wiley & Sons Inc., 1995) at pages 2.9.1 through 2.9.20, which is herein incorporated by reference.

According to such methods, Southern blotting involves separating DNA molecules according to size by gel electrophoresis, transferring the size-separated DNA to a synthetic membrane, and

hybridizing the membrane bound DNA to a complementary nucleotide sequence labelled radioactively, enzymatically or fluorochromatically. In dot blotting and slot blotting, DNA samples are directly applied to a synthetic membrane prior to hybridization as above.

An alternative blotting step is used when identifying complementary nucleotide sequences in a cDNA or genomic DNA library, such as through the process of plaque or colony hybridization. A typical example of this procedure is described in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL 2nd Ed. (Cold Spring Harbour Press, 1989) Chapters 8-12 which are herein incorporated by reference.

Typically, the following general procedure can be used to determine hybridization conditions. Nucleotide sequences are blotted/transferred to a synthetic membrane, as described above. A wild type nucleotide sequence of the invention is labelled as described above, and the ability of this labelled nucleotide sequence to hybridize with an immobilized nucleotide sequence analyzed.

A skilled addressee will recognize that a number of factors influence hybridization. The specific activity of radioactively labelled polynucleotide sequence should typically be greater than or equal to about 10^8 dpm/mg to provide a detectable signal. A radiolabelled nucleotide sequence of specific activity 10^8 to 10^9 dpm/mg can detect approximately 0.5 pg of DNA. It is well known in the art that sufficient DNA must be immobilized on the membrane to permit detection. It is desirable

to have excess immobilized DNA, usually 10 µg. Adding an inert polymer such as 10% (w/v) dextran sulfate (MW 500,000) or polyethylene glycol 6000 during hybridization can also increase the sensitivity of hybridization (see Ausubel *supra* at 2.10.10).

To achieve meaningful results from hybridization between a nucleotide sequence immobilized on a membrane and a labelled nucleotide sequence, a sufficient amount of the labelled nucleotide sequence must be hybridized to the immobilized nucleotide sequence following washing. Washing ensures that the labelled nucleotide sequence is hybridized only to the immobilized nucleotide sequences with a desired degree of complementarity to the labelled nucleotide sequence.

"Stringency" as used herein, refers to the temperature and ionic strength conditions, and presence or absence of certain organic solvents, during hybridization. The higher the stringency, the higher will be the degree of complementarity between the immobilized nucleotide sequences and the labelled polynucleotide sequence.

"Stringent conditions" designates those conditions under which only nucleotide sequences having a high frequency of complementary bases will hybridize.

Typical stringent conditions are (1) 0.75 M dibasic sodium phosphate/0.5 M monobasic sodium phosphate/1 mM disodium EDTA/1% sarkosyl at about 42°C for at least 30 minutes; (2) 6.0 M urea/0.4 % sodium lauryl sulfate/0.1x SSC at about 42°C for at least 30 minutes; (3)

0.1x SSC/0.1% SDS at about 68°C for at least 20 minutes; (4) 1x SSC/0.1% SDS at about 55°C for about 60 minutes; (5) 1x SSC/0.1% SDS at about 62°C for about 60 minutes; (6) 1x SSC/0.1% SDS at about 68°C for about 60 minutes; (7) 0.2X SSC/0.1% SDS at about 55°C for about 60 minutes; (8) 0.2x SSC/0.1% SDS at about 62°C for about one hour; and (9) 0.2X SSC/0.1% SDS at about 68°C for about 60 minutes. For a detailed example, see CURRENT PROTOCOLS IN MOLECULAR BIOLOGY *supra* at pages 2.10.1 to 2.10.16, and Sambrook et al in MOLECULAR CLONING. A LABORATORY MANUAL (Cold Spring Harbour Press, 1989) at sections 1.101 to 1.104, which are hereby incorporated by reference.

While stringent washes are typically carried out at temperatures from about 42°C to 68°C, one skilled in the art will appreciate that other temperatures may be suitable for stringent conditions. Maximum hybridization typically occurs at about 20° to 25° below the T_m for formation of a DNA-DNA hybrid. It is well known in the art that the T_m is the melting temperature, or temperature at which two complementary polynucleotide sequences dissociate. Methods for estimating T_m are well known in the art (see CURRENT PROTOCOLS IN MOLECULAR BIOLOGY *supra* at page 2.10.8). Maximum hybridization typically occurs at about 10° to 15° below the T_m for a DNA-RNA hybrid.

Other stringent conditions are well-known in the art. A skilled addressee will recognize that various factors can be manipulated to

optimize the specificity of the hybridization. Optimization of the stringency of the final washes can serve to ensure a high degree of hybridization.

Methods for detecting labelled nucleotide sequences hybridized to an immobilized nucleotide sequence are well known to practitioners in the art. Such methods include autoradiography, chemiluminescent, fluorescent and colorimetric detection.

In yet another aspect, the invention provides a pharmaceutical composition for alleviating blood loss in patients, said composition comprising the peptides, peptide homologs, derivatives or analogues of the invention (*"therapeutic agents"*) suitably in the form of a pharmaceutically acceptable salt.

The term "pharmaceutically acceptable salt" as used herein refers to a salt which is toxicologically safe for human and animal administration. This salt may be selected from a group including hydrochlorides, hydrobromides, hydroiodides, sulphates, bisulphates, nitrates, citrates, tartrates, bitartrates, phosphates, malates, maleates, napsylates, fumarates, succinates, acetates, terephthalates, pamoates and pectinates.

According to yet another aspect of the invention, there is provided a method for alleviating blood loss in patients, said method comprising the step of administering to a patient in need of such treatment a therapeutically effective dosage of a therapeutic agent of the invention in the form of a pharmaceutically acceptable salt.

Any suitable route of administration may be employed for providing a human or lower animal the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intramuscular, intradermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal and the like may be employed. Preferably, an intravenous route is employed.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Pharmaceutically-acceptable carriers for systemic administration may also be incorporated into the compositions of this invention.

By "pharmaceutically-acceptable carrier" is meant a solid or liquid filler, diluent or encapsulating substance which may be safely used in systemic administration. Depending upon the particular route of

administration, a variety of pharmaceutically-acceptable carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline, and pyrogen-free water.

Pharmaceutical compositions of the present invention suitable for oral or parenteral administration may be presented as discrete units such as capsules, sachets or tablets each containing a pre-determined amount of one or more therapeutic agents of the invention, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more therapeutic agents as described above with the carrier which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the therapeutic agents of the invention with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation.

The above compositions may be administered in a manner compatible with the dosage formulation, and in such amount as is therapeutically effective to alleviate blood loss in patients. The quantity of

the therapeutic agent(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof. In this regard, precise amounts of the therapeutic agent(s) required to be administered will depend on the judgement of the practitioner. However, suitable dosages are readily determined by those of skill in the art and may be in the order of nanograms to micrograms of the therapeutic agents of the invention.

In a still further aspect, the invention resides in an anti-tumour agent comprising a peptide, peptide homolog, derivative or analogue according to the invention conjugated with an anti-fibrin antibody. Such a conjugate may be useful in targeting fibrin and quenching plasmin at the immediate site of tumours to thereby inhibit progression and invasiveness of such tumours. Reference may be made in this regard to an abstract by Raut and Gaffney (1996, *Fibrinolysis* **10** (Suppl. 4):1-26, Abstract No 39) which is hereby incorporated by reference.

The anti-fibrin antibodies may include any suitable antibodies which bind to or conjugate with fibrin, preferably human fibrin. For example, the anti-fibrin antibodies may comprise polyclonal antibodies. Such antibodies may be prepared for example by injecting fibrin into a production species, which may include mice or rabbits, to obtain polyclonal antisera.

In lieu of the anti-fibrin polyclonal antisera obtained in the

production species, monoclonal antibodies may be produced using the standard method as for example, described in an article by Köhler and Milstein (1975, *Nature* **256**:495-497) which is hereby incorporated by reference, or by more recent modifications thereof as for example, described in "CURRENT PROTOCOLS IN IMMUNOLOGY" (1994, Ed. J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach and W. Strober, John Wiley and Son Inc. which is hereby incorporated by reference) by immortalising spleen or other antibody producing cells derived from a production species which has been inoculated with fibrin.

Preferred monoclonal antibodies which may be used to produce the anti-tumour agent of the invention include, but are not limited to, the anti-fibrin monoclonal antibodies disclosed by Tymkewycz *et al* (1993, *Blood Coagul. Fibrinol.* **4**:211-221) which is hereby incorporated by reference or the monoclonal antibody described by Raut and Gaffney (1996, *supra*).

Also contemplated are anti-fibrin antibodies which comprise Fc or Fab fragments of the polyclonal or monoclonal antibodies referred to above. Alternatively, the anti-fibrin antibodies may comprise single chain Fv antibodies (scFvs) against the peptides of the invention. Such scFvs may be prepared, for example, in accordance with the methods described respectively in United States Patent No 5,091,513, European Patent No 239,400 or the article by Winter and Milstein (1991, *Nature* **349**:293) which are hereby incorporated by reference.

Any suitable procedure may be used to conjugate the anti-fibrin antibodies with a peptide, peptide homolog, derivative or analogue according to the invention. For example, reference may be made to the 'zero-length' cross linking procedure of Grabarek and Gergely (1990, *Anal. Biochem.* **185**:131-135) which is hereby incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

In order that the invention may be readily understood and put into practical effect, preferred embodiments will now be described by way of example with reference to the accompanying drawings in which:

FIG. 1 refers to a Sephacryl S-300 elution profile of venom from Australian brown snake;

FIG. 2 shows a DEAE-Sepharose CL-6B column elution profile of the concentrated plasmin inhibitor activity derived from the Sephacryl S-300 chromatography in FIG. 1;

FIG. 3 shows a Sephacryl S- 100 elution profile of one of the two pooled and concentrated fractions from the DEAE-Sepharose CL-6B chromatography;

FIG. 4 refers to real time curve fit analysis, using Sigmaplot, of Txln 1 (0- 41 0 nM) inhibition of plasmin (2 nM) ;

FIG. 5 lists the amino acid sequences for TXLN 1 and TXLN 2, as well as those of Taicotoxin associated plasmin inhibitor (TAC) and aprotinin (APRO);

FIG. 6 lists the cDNA sequence of *txln 1* encoding Txln 1;

and

FIG. 7 lists the cDNA sequence of *txln 2* encoding Txln 2.

EXAMPLES

EXAMPLE 1

Characterization of two plasmin inhibitors from Pseudonaja textilis textilis which inhibit bleeding in an animal model

MATERIALS AND METHODS

Materials

Pooled dried *P. textilis* venom was obtained from Mr Peter Mirtschin, Venom Supplies, Tanunda, South Australia. Venom was reconstituted in 0.05 M Tris-HCl buffer pH 7.4, 10 mg/mL and the solution was centrifuged (2000xg; 30 min) before chromatography or analysis. Sephacryl S-300, Sephacryl S-100, con A-Sepharose and DEAE-Sepharose CL-6B were obtained from Pharmacia Uppsala, Sweden, and the synthetic chromogenic substrates S-2222,

S-2251, S-2160 and S-2238 were from Kabi Diagnostica, Stockholm, Sweden. Human plasminogen was purified from outdated pooled citrated plasma using the method of Deutsch and Mertz, 1970, Human plasmin was prepared from plasminogen by activation with urokinase-bound Sepharose 4B as described by Robbins, 1978 and calibrated against an International Standard for plasmin (77/558). Kinetic experiments were conducted using a highly purified plasmin from Choay Laboratories, Paris. All other buffers and reagents were AnalR grade.

Plasmin Inhibitory Assay

The plasmin inhibitory assay mixture was made up of 0. 90 mL of 0. 15 M Tris-HCl, pH 7.4, 0.025 mL (0. 1 IU) of plasmin, 0.025 mL of inhibitor to which is added 0.050 mL of 3.0 mM substrate S-2251. Residual plasmin was assayed using the chromogenic substrate, S-2251, with a Hitachi 557 recording spectrophotometer at 405 nm. The rate of hydrolysis was monitored continuously. A standard curve of plasmin activity was prepared using the International standard (77/558).

Chromatography

Purification of TxIn 1 and 2

Sephacryl S-300 column (5.0 x 95 cm) was equilibrated with 0. 1 M ammonium acetate pH 7.0 at a flow rate of 1 mL per minute. 500 mg of lyophilised *P. textilis* venom was reconstituted in 25 mL of column buffer, centrifuged using a Beckman centrifuge at 10,000 LKB fraction collector, in time-base mode and the eluate was monitored at 280 nm using an Altex dual wavelength in line UV detector. The pooled plasmin inhibitor fractions were concentrated using an Amicon stirred cell concentrator Model 402, using a YM 3 membrane and this concentrate was applied to DEAE-Sepharose column. DEAE-Sepharose column (2.5 x 12 cm) was equilibrated with 0.05 M phosphate buffer pH 8.0 at a flow rate of 1.0 mL per minute. Following the application of the concentrated plasmin inhibitor, the column was washed with buffer giving a non-bound protein peak with no plasmin inhibitory activity. A linear gradient of NaCl

(0-0.5 M, 500 mL) was applied at a flow rate of 1.0 mL per minute. Sephacryl S-100 column (2.5 x 95 cm) was equilibrated with 0.05 M Tris-HCl, pH 7.4. Pooled plasmin inhibitors TxIn 1 and TxIn 2 (concentrated on Amicon 402 stirred cell using YM 3 membrane) were individually further purified on this column. Fractions with the highest plasmin inhibitory activity were pooled, concentrated and stored at concentrations of about 1 mg/mL (approx. 143 µM) in 50 % glycerol/ saline. Con A-Sepharose affinity chromatography column (1 x 10 cm) was equilibrated with 0.15 M Tris-HCl buffer pH 7.4. The pooled and concentrated plasmin inhibitor was applied at a flow rate of 1.0 mL per minute and the inhibitory activity was found in the wash peak.

Reverse-phase chromatography was used as an analytical procedure and was carried out using a Waters C_{18} µbondpack column (0.6 x 30 cm), equilibrated with 0.05 % trifluoroacetic acid (TFA) in water and developed using a 0% to 70 % acetonitrile gradient. The chromatography was monitored at 214 nm and the gradient was developed over 60 minutes.

SDS-PAGE was essentially by the method of Weber and Osborn (1969, *J. Biol. Chem.* **244**:4406-4409). Samples were prepared by the method of Dobos and Gaffney (1971, *FEBS Letters* **15**:13-15) which incorporates 4 M urea in the sample solution.

Amino Acid Sequencing

TxIn 1 and 2 were reduced in 6 M guanidine hydrochloride.

The sequencing of TxIn 1 and 2 was carried out using a Hewlett Packard G10005 A sequencer, first carrying out a long N-terminal sequencing, followed by both endoLys C and endoproteinase Asp N digestion of purified TxIn 1 and 2, as well as rp-8 reverse-phase chromatography of the digest to isolate the various fragments. The evidence for the sequence was essentially derived from (1) a long N-terminal sequence run of the whole molecule, (2) an extended sequence of an endoLys C peptide obtained by further chromatography of one of the peptides isolated by reverse-phase chromatography, (3) the sequence of an endoproteinase Asp N peptide and (4) on mass spectroscopy data on both native molecule and the reduced, carboxymethylated TxIn 1 and 2. It is of interest that the Asp-N digest lead to a cleavage of a Pro-Asp bond which is quite unusual.

Chromatography of samples for sequencing

Reverse-phase high pressure liquid chromatography (HPLC) was carried out on a Hewlett Packard 1090 liquid chromatograph using a flow rate 0.2 mL/minute. Linear gradients were formed between buffer A and buffer B where A was 0. 1 % TFA and buffer B was 0. 1% TFA in 70% acetonitrile; the absorbance at 214 nm of buffer A was carried out at room temperature.

Reduction and Carboxymethylation

TxIn 1 and 2 were reduced in 6 M guanidine hydrochloride, 0. 1 M Tris chloride, 1 mM EDTA, pH 9.5 with 10 mM DTT for 2 hours

under Argon at 37°C and carboxymethylated with 15 mM iodoacetic acid for 30 minutes. Reduced and carboxymethylated TxIn 1 and 2 were isolated by reverse-phase HPLC.

Digestion with Endoproteinase Asp N

TxIn 1 and 2 were digested with endoproteinase Asp N in 50 mM sodium phosphate buffer, pH 8.0 at 37°C for 18 hours. The reaction was stopped by acidification with TFA and subjected to RP-HPLC.

Digestion with endoproteinase Lys C

TxIn 1 and 2 were digested with endo proteinase Lys C in 50 mM phosphate buffer, pH 8.0 at 37°C for 18 hours, using an enzyme to substrate ratio of 1:100. The reaction was stopped with TFA and subjected to RP-HPLC.

Sequencing

Amino acid sequence determination was carried out on a Hewlett Packard G10005A sequencer.

Mass spectrometry

Matrix-assisted laser desorption/ionization (MALDI) time of flieght (TOF) mass spectrometry was performed with a Bruker Reflex mass spectrometer (Bruker-Franzen Analytik, GMBH Breman Germany) operated exclusively in the reflectron mode. Samples were diluted 30% in aqueous acetonitrile containing 0.1% TFA and 2 µL of a matrix comprised of 2,6-dihydroacetophenone containing diammonium hydrogen citrate prior to deposition of 0.5 -1 µL onto a stainless steel target.

Mouse tail vein bleeding model

A bleeding model was established using mature outbred Quackenbush mice (average 20 g), of both sexes after anaesthesia was induced by intra peritoneal injection of 0.4 mL of a one in ten dilution of an equal volume mixture of Ketamine (100 mg/mL) and Rompun (xylazine, 40 mg/mL). Blood loss was measured following a clean excision of the tail, The blood was collected into a preweighed Eppendorf™ tubes. Accuracy dictated that blood loss was measured by weight rather than volume. All mice were euthanized by cervical dislocation.

RESULTS

Purification

FIG. 1 shows the Sephacryl S-300 chromatographic separation of proteins from the crude venom showing three major and two minor peaks of protein, labelled 1-5. Plasmin inhibitory activity is indicated in the right-hand shoulder of peak three (see shaded area), using the plasmin neutralization assay to monitor the eluted fractions. Further fractionation of the pooled inhibitor fractions, (Amicon YM3 concentrated), was performed on a DEAE-Sepharose CL-6B column. FIG. 2 shows the resultant separation, indicating two distinct peaks of plasmin inhibitory activity, denoted TxIn 1 and TxIn 2. Each peak was pooled separately, concentrated and applied to a Sephacryl S-100 column to remove trace impurities. FIG. 3 shows the elution profile of TxIn 1 which is identical to that of TxIn 2, however the insert in FIG. 3 shows the

reverse-phase HPLC profiles of each TxIn indicating each to have a distinct elution volume from this column. The purity of the S-100 eluted material was further demonstrated by SDS-PAGE gel electrophoresis (data not shown). The final concentrated plasmin inhibitors were stored at -20°C in 0.05 M Tris buffered saline at a final concentration of about 1 mg/mL.

While these preparations were adequate for kinetic and physical characterization, it was noted that they caused distress in the mouse model used to assess bleeding. For such experiments, it was necessary to remove trace amount of a potent prothrombin activator complex using a Con A-Sepharose column (Masci *et al*, 1988, *Biochem. Intern.* **17**:825-835).

FIG. 4 shows real time rates of plasmin activity using a 2 nM plasmin concentration in the presence of no inhibitor and increasing concentration of TxIn 1. These data are also representative of TxIn 2. The Ki's for TxIn 1 and 2 were determined using curve-fit analysis of the resultant inhibition curves applied to Sigmaplot programme. The Ki's determined for TxIn 1 and 2 were 3.5 nM and 2.0 nM (n=6) respectively. Using a pooled mixture of partially purified TxIn 1 and TxIn 2, the Ki was found to be 13.9 nM. ($N = 3$). This data is summarized in TABLE 2.

Physical characterization

Primary Sequence

FIG. 5 shows a comparison of the amino acid sequences of

TxIn 1 and 2 with those of aprotinin and Taicotoxin-associated plasmin inhibitor isolated from the venom of the Australian Eastern Taipan, *Oxyuranus scutellatus* (having the closest homology to TxIn 1 and 2). It can be seen that all four plasmin inhibitors have the disulphide bond arrangement which is typical of this group of plasmin inhibitors and endows them with great stability. It was found that TxIn 1 and 2 could be heated at 80°C for two hours with no loss of inhibitory activity. A sequence difference of seven amino acids was observed between TxIn 1 and 2, while each showed respectively 49 and 47 % homology with Aprotinin and Taicotoxin-associated plasmin inhibitor. It was observed that there was almost 100 % homology between Bovine lung-source Aprotinin and Taipan plasmin inhibitor. Both TxIn 1 and TxIn 2 are quite acidic proteins with nett negative charges of -4 (TxIn 1) and -6 (TxIn 2), while both Aprotinin and Taicotoxin are quite basic, each having a nett charge of +6.

Molecular weight

Mass spectroscopy data for TxIn 1 and 2 showed molecular weight of 6682.4 and 6689.3 respectively (data not shown), while the molecular weight from the amino acid composition were 6682.4 and 6692.4 respectively. Note that this allows us to assign the final residue as a serine; the actual sequence information finishes with Gly 58.

Behaviour of TxIn 1 and TxIn 2 in an animal bleeding model

The effect of intravenous TxIn 1 and 2 on the blood-loss

from an excised mouse tail vein is shown in TABLE 3 and for comparison the results of Aprotinin is shown. The amount used was equivalent on a weight basis to the amount of Aprotinin used clinically in humans. This amounted to 140 µg (20 nM) of each substance studied per 20 gram mouse. It can be seen from TABLE 3 that Aprotinin reduced blood loss by 60 % while both TxIn 1 and TxIn 2 reduced blood loss 50%.

DISCUSSION

Reduction in blood flow during major surgery and following trauma is of concern today because of a deteriorating blood donor status. The increased incidence of viral contamination of blood has introduced socio-medical problems which do not seem to abate. There is anxiety concerning the contamination of blood by HIV, hepatitis B and C viruses, while the potential for cross-contamination by prions associated with Bovine Spongiform Encephalitis (BSC) and Creutzfeldt-Jakob's disease remains a major cloud over the whole blood transfusion area.

Aprotinin is used as derived from bovine lung for the stemming of blood flow during surgical procedures such as cardiopulmonary bypass (CPB) (for review, see Royston, 1992, *J. Cardiothorac. Vasc. Anest.* **6**:76-100). Indeed, while CPB is the major surgical circumstance in which aprotinin is used, blood loss during neurosurgery (Guidetti and Spallone, 1981, *Surg. Neurol.* **15**:239-246), orthopaedic (Ketterl *et al*, 1982, *Medizin Welt* **33**:480-486), liver (Neuhaus *et al*, 1989, *Lancet* **ii**:924-925), and urological (Kosters *et al*, 1973,

Urologe **12**:295-296) surgeries have been reduced using this drug.

In order to provide alternative haemostatics based on plasma inhibition, snake venoms have been studied for some years. The first report of a plasmin/ trypsin inhibitor found in snake venom was by Takahashi *et al* (1972, *FEBS Letters* **27**:207-210) while there are further reports of plasmin inhibitors in other viper and elapid venoms (Shafqut *et al*, 1990, *Eur. J. Biochem.* **194**:337-341; Shafqut *et al*, 1990, *FEBS. Lett.*, **27**568; Yamakawa *et al*, 1987, *Biochem. Biophys. Acta.* **925**:124-132; Ritonja *el al*, 1983, *Eur. J. Biochem.* **133**:427-432; Strydom *et al.* 1979, *Biochem Biophys. Acta.* **491**:361-369; Takahashi *et al.* 1974, *Toxicon.* **12**:193-197). Further screening of Australian elapid venoms has shown that two snake genera possess potent plasmin inhibitors (Masci, 1986, *The effects of Australian snake venoms on coagulation and fibrinolysis.* Masters thesis. University of Queensland). These are the *Pseudonaja and Oxyuranus* genera. In the *Pseudonaja* genus, the venom from all species were shown to possess an inhibitor of plasmin. This has been isolated and kinetically characterised from the Textilinin subspecies (Willmott *et al*, 1995, *supra*) and named TEXTILININ (TXLN). Further purification (FIGS. 1-3) has shown that there are two forms of this inhibitor, Textilinin 1 and 2. In the *Oxyuranus* genus, the venom of only one species was shown to contain a plasmin/trypsin inhibitor and Possani *et al* (1992, *Toxicon.* **30**:1343-1364) have reported the sequence of this inhibitor and have shown it to be associated with a multimeric complex.

This complex was demonstrated to be a calcium channel blocker containing alpha neurotoxin, a phospholipase and the trypsin inhibitor and was called Taicotoxin. FIG. 5 shows that this trypsin inhibitor (TAC) has 65 % homology with Textilinin 1, the closest homology to Textilinin of the known naturally occurring plasmin inhibitors.

While studying the kinetics of a partially purified preparation of *P. Textilis* venom, it had been observed that the plasmin inhibitor which we named TxIn bound rapidly and more specifically to plasmin than did aprotinin (Willmott *et al*, 1995, *supra*). The results also showed with this preparation that the binding was less tight (150 nM) than aprotinin (53 pM) for plasmin. The specificity of aprotinin was shown by Willmott *et al* (1995, *supra)* to be broad based, neutralizing tPA, urokinase and kallikrein, as well as plasmin and trypsin. Studies by the same investigators on the snake venom plasmin inhibitor TxIn showed that TxIn binds more specifically to plasmin and trypsin in a rapid single step reaction which seems to be reversible. Since aprotinin has been reported to be associated with increased incidence of vein-graft occlusion (Van der Meer, 1996, *supra*) and thrombosis (Sumama *et al*, 1994, *supra*; Cosgrove *et al*, 1992, *supra*), it was postulated that a less-tight binding inhibitor such as TxIn may be of greater clinical efficacy. This original finding had prompted us to further purify the TxIn from the venom and it was then found that each snake venom contained two forms of the TxIn, as mentioned above. This reflects the work of Takahashi *et al* (1972,

supra), who also reported two variants of a Russels viper venom plasmin inhibitor. Both TxIns bound to plasmin less tightly than aprotinin, confirming the earlier data for the partially purified preparation (see TABLE 2). FIG. 5 shows that there is a 7 amino acid difference between TxIns 1 and 2, and that both are acidic, containing nett negative charges (-4 and -6 respectively), as distinct from aprotinin which a basic molecule (+6).

Both TxIns (1 and 2) reduce blood loss in a mouse tail-vein bleeding model (TABLE 3) as efficaciously as aprotinin in the same model. If the reduction in blood loss in this model is associated with plasmin neutralization at the site of the haemostatic plug formation as suggested (Orchard *et al*, 1993, *supra*), it is not surprising that they compare favourably. The inability of TxIn to neutralize kallikrein in contrast to aprotinin may have some clinical significance. This of course depends on the contribution of the kallikrein - factor XIII pathway on the production of plasmin at the site of wound-healing (Kluff *et al*, 1987, *Sem. Thromb. Haemost.* **13**:50-68). Indeed, the kallikrein inhibitory effect of aprotinin may be a contributing factor in the prethrombotic claims for this drug.

What role the TxIn molecule plays in the human coagulation imbalance associated with snake bite is unclear since envenomation is accompanied by a dramatically increased fibrinolytic activity which is in turn related to it disseminated intravascular coagulation in the bitten

individual. Presumably this fibrolitic activity is stimulated by the prothrombin-mediated fibrin complex (de Serrano *et al*, 1988, *J. Prot. Chem.* **8**:61-77). That the subsequent inhibition of fibrinolysis might contribute to this fibrin-mediated occlusion of the microvasculature is arguable.

With a view to answering some of the questions concerning the physiological behaviour of TxIns, both in arresting haemorrhage and controlling plasmin activity in a variety of pathologies, we have cloned cDNA for each of TxIn 1 and TxIn 2 with a view to producing a recombinant form of these plasmin inhibitors for further studies. In view of the kinetic profile and a narrow specificity of the TxIns, these plasmin inhibitors are considered to have potential clinical advantage over aprotinin in connection with rebound thrombosis.

EXAMPLE 2

Cloning and Sequencing of Textilinin cDNA

Isolation of total RNA

Total RNA was isolated using the Dynal Bead total RNA extraction kit. The source of tissue used for this isolation was venom glands which were surgically removed under sterile conditions from animals which were deemed to be euthanazed due to lethal damage by vehicles or required to be destroyed due clinical conditions. Frozen venom glands (2) were placed in 1.0 mL of lysis buffer (supplied in the kit) in an Eppendorf™ tube and immediately homogenized using a RNAse-

free sterile Polytron probe. Homogenization was carried on ice in 4x10 second intervals.

The homogenate was divided in 0. 5 mL aliquots and an equal volume phenol-chloroform (1:1) extraction carried out. The aqueous layer (top) was separated which contained RNA and DNA which was precipitated with an equal volume of isopropanol overnight. After centrifugation at 13,000 rpm for 20 minutes at 4°C, a 70% ethanol washing was carried out. The precipitated RNA was reconstituted in DEPC treated water and nucleic acid content determined on diluted aliquot by measurement of absorbance at 260 nm.

Isolation of mRNA

Messenger RNA was isolated using the Dynal Magnetic Beads as recommended by supplier. After elution of mRNA from magnetic beads, 1 µg was used for reverse-transcriptase (RT) polymerase chain reaction (PCR) and remainder was precipitated in one tenth volume of 3 M sodium acetate pH 5.2/ 2 volumes of absolute ethanol and stored at -70°C.

RT-PCR

Reverse transcriptase (RT) PCR was carried using Promega RT kit MMLV reverse transcriptase and the isolated mRNA as template at 42°C for 1 hour. The resulting cDNA was used for second strand synthesis. Second strand synthesis was carried out using T4 DNA polymerase and first strand cDNA as template. The reaction was carried

at 14°C for 3 hours. Final volume of second synthesis reaction was 100 µL. Phenol-chloroform extraction was carried out and aqueous layer (top, containing double stranded cDNA) was transferred into a clean Eppendorf™ and cDNA was precipitated with ethanol overnight. After centrifugation at 13,000 rpm for 20 minutes at 4°C, the resulting precipitate was washed with 70% ethanol and reconstituted in 10 µL of sterile water and stored frozen at -20°C until used in PCR amplification *txln* genes using degenerate primers to *txln 1* and *txln 2*.

Txln cDNA-PCR

Degenerate primers synthesized:

Masci-3 (sense) 5'-ATGAA(AG)GA(CT)AG(AG)CC(ACT)GA (AG)(CT)T(AGCT) GA(AG)-3'

Masci-4 (sense) 5'-ATGAA(AG)GA(TC)(AC)GNCCNGA(TC)-3'

Not-Nco (sense) 5'-AATACCATGGCGGCCGCTCACACCCAAGCTTTCC-3'

Masci-5 (antisense) 5'-GT(AG)CT(TC)TC(AG)TG(TC)TC(TC)TC(CT)-3'

Masci-6 (antisense) 5'-GT(AG)CT(TC)TC(AG)CA(TC)TC(TC)TC-3'

Degenerate oligonucleotides primers (sense at 5' end and antisense at 3' end) have been designed from the amino acid sequences of *txln 1* and *txln 2* in order to perform PCR using cDNA as a template. Genomic DNA was isolated from the liver tissue of the Brown Snake and was also used as template in PCR using degenerate primers to determine intron sequences of the *txln* genes. Using cDNA, a number of PCR

conditions were used which finally resulted in a number of products, one being 180 base pairs which was of the expected size, corresponding to a gene coding for 60 amino acids . Similarly, using genomic DNA a 180 base pair product was obtained.

PCR amplification parameters used were 94°C for 1 minute; 40°C for 1 minute; 72°C for 1 minute.

A number of PCR reaction were carried out and PCR products was separated on 2 % TAE agarose gel. A DNA band migrating at about 180 base pair was excised and gel purified using phenol-chloroform extraction and ethanol precipitation.

The 180 base pair PCR products corresponding to *txln 1* and *txln 2* were cloned into p-GEM 5zf (Promega) and the resulting recombinant plasmids were used as templates for automated nucleotide sequence analysis. The respective nucleotide sequences relating to the coding region of *txln 1* and *txln 2* are shown in FIGS. 6 and 7.

EXAMPLE 3

Production of a fibrin-specific monoclonal antibody-textilinin 1 construct

A fibrin specific monoclonal antibody, MAb 12B3.B10 (IgG2A/kappa) (Tymkewycz *et al*, 1993, *supra*), will be chemically conjugated with the plasmin inhibitor Txln 1 by a two step zero length crosslinking procedure according to Grabarek and Gergely (1990, *Anal. Biochem.* **185**:131-135). Briefly Txln 1 will be incubated with a water

soluble carbodiimide (EDC) in the presence of N-Hydroxysuccinimide (sulfo-NHS), and will result in the conversion of the carboxyl groups of Glu or Asp into succinimidyl esters. After removing excess EDC by gel filtration MAb 12B3.B10 will be added to the activated TxIn 1. Crosslinking will result from nucleophilic substitution of the lysine-amino groups of the IgG for the succinimidyl moieties during a 2h incubation. The IgG-TxIn 1 conjugate will then purified from free TxIn 1 via size exclusion HPLC on a Superdex 200 HR 10/30 column as described by Raut and Gaffney (1996, *Fibrinolysis* **10** (Suppl. 4):1-26, Abstract No 39). The purified construct will then be tested for plasmin inhibitory activity by ELISA using the Chromogenic substrate S-2251.

DATED this eleventh day of May, 1998

THE UNIVERSITY OF QUEENSLAND

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NATIONAL INSTITUTE OF BIOLOGICAL STANDARDS AND CONTROL, UNITED KINGDOM

By their Patent Attorneys

FISHER ADAMS KELLY

TABLES

TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisoleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamate	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmom
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-medlylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap

TABLE 1 cont'd

	D-α-methylasp	Dmasp	α-methylpenicillamine	Mpen
	D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
5	D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
	D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Nom
	D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
	D-α-methylleucine	Dmleu	α-napthylalanine	Anap
	D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
10	D-α-methylmethionine	Dmmet	N-(2-carbamylediyl)glycine	Ngln
	D-α-methylornithiine	Dmom	N-(carbamylmethyl)glycine	Nasn
	D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
15	D-α-methylthreo	Dmthr	N-cycloheptylglycine	Nchep
	D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
	L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
	L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
20	L-α-methylnorvatine	Mnva	L-α-methylornithine	mom
	L-α-methylphenylalanine	Mphe	L-α-methylpmline	Mpm
	L-α-methylserine	Mser	L-α-methylthreonine	Mthr
	L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
	L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
25	N-(N-(2,2-diphenylethyl carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl carbamylmethyl)glycine	Nnbhe
	1-carboxy-1-(2,2-diphenyl-ethyl amino)cyclopropane	Nmbc		

TABLE 2

Ki (nM)		
Plasmin Concentration	2 nM (n=6)	18 nM (n=6)
S 100 Pool (TxIn 1 and 2)	7.1 ± 0.2	13.9 ± 0.3
TxIn 1	3.5 ± 3	2.6 ± 0.2
TxIn 2	2.2 ± 0.2	2.8 ± 0.3

TABLE 3

	blood weight (gms) (N=6)	% control
Control	0.869 ± 0.004	100
aprotinin (1000 units)	0.352 ± 0.004	40.5
Txln 1 (1000 units)	0.528 ± 0.006	60.8
Txln 2 (1000units)	0.502 ± 0.004	57.8

TABLE LEGENDS

TABLE 1. *Unconventional amino acids for generation of modified peptides.*

TABLE 2. *Summary of inhibitory constants.* Ki for Txln S-100 Pool measured using Enzfitter analysis programme, using plasmin concentration 0. 5 nM was 0. 15 μM (n = 6).

TABLE 3. *Mouse tail bleeding model - Blood loss determination.*

FIGURE LEGENDS

FIG. 1. Sephacryl S-300 elution profile of venom from Australian brown snake. Five protein peaks (1-5) were obtained and plasmin inhibitory activity (e.g. TxIn) was obtained on the shoulder peak 4 which comprises about 2% of the total protein applied to the column.

FIG. 2. DEAE-Sepharose CL-6B column elution profile of the concentrated plasmin inhibitor activity derived from the Sephacryl S-300 chromatography in FIG. 1. The solid bars show two separate peaks of plasmin inhibitory activity (denoted 1 and 2).

FIG. 3. Sephacryl S-100 elution profile. One of the two pooled and concentrated fraction from the DEAE-Sepharose CL-6B chromatography. The profile shown is that of TxIn 1 but the profile of TxIn 2 is identical. Insert, however shows two distinct elution profiles for each of TxIn 1 and TxIn 2 using reverse-phase C 18 HPLC chromatography.

FIG. 4. Real time curve fit analysis using Sigmaplot of TxIn 1 (0-410 nM) inhibiton of plasmin (2 nM). Similar inhibition curves (data not shown) were obtained with TxIn 2.

FIG. 5. Amino acid sequences for TXLN 1 and TXLN 2, as well as those of Taicotoxin associated plasmin inhibitor (TAC) and aprotinin

(APRO). The sequences were aligned according to the location of the six cysteines.

FIG. 6. cDNA sequence of *txln 1*. The amino acid sequence encoded by *txln 1* is shown below the nucleotide sequence in single letter code. The letter "N" denotes a non-characterized nucleotide.

FIG. 7. cDNA sequence of *txln 2*. The amino acid sequence encoded by *txln 2* is shown below the nucleotide sequence in single letter code. The letter "N" denotes a non-characterized nucleotide.

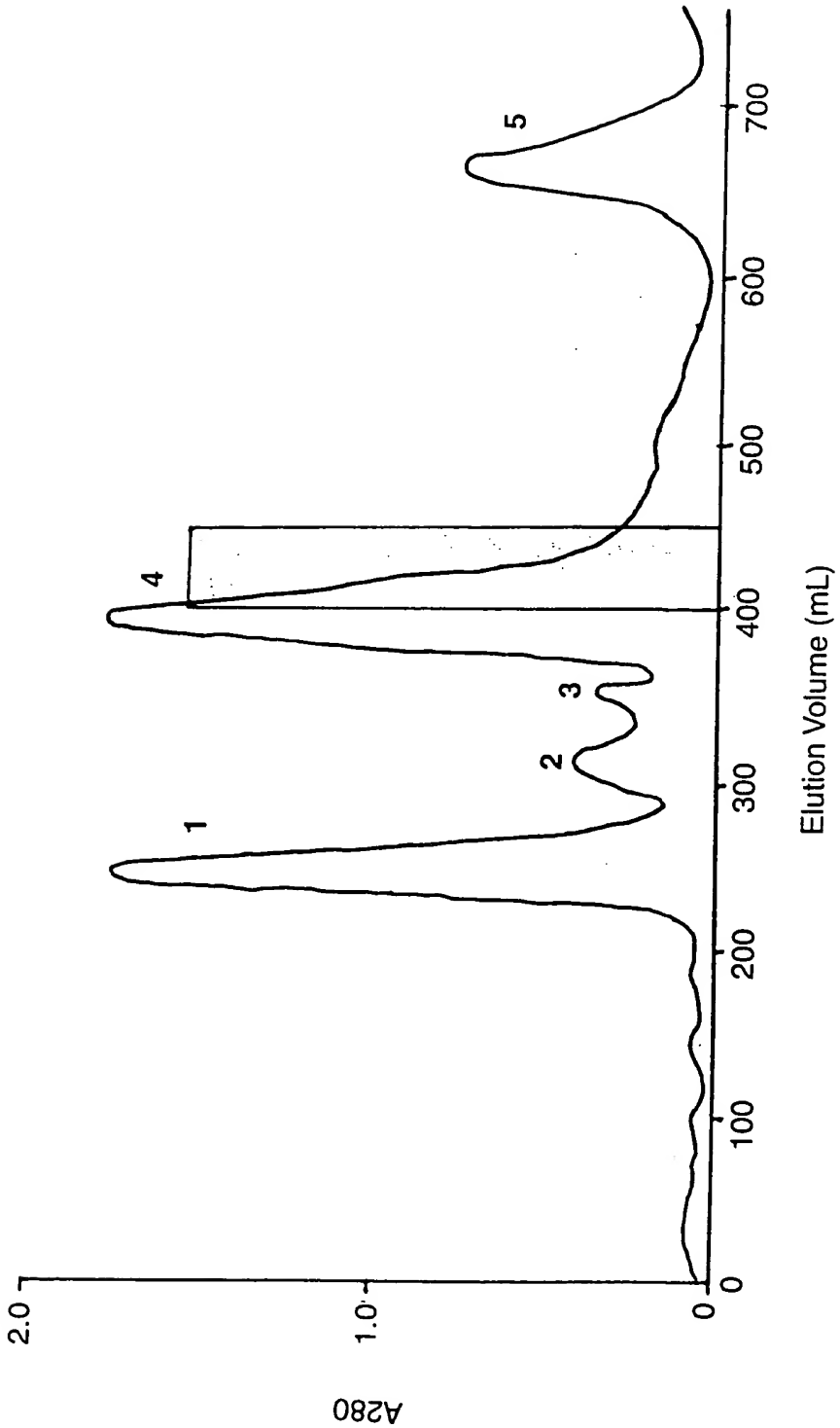
A280
2.0
1.0
0
0
100
200
300
400
500
600
700
Elution Volume (mL)
1
2
3
4
5

FIG. 1

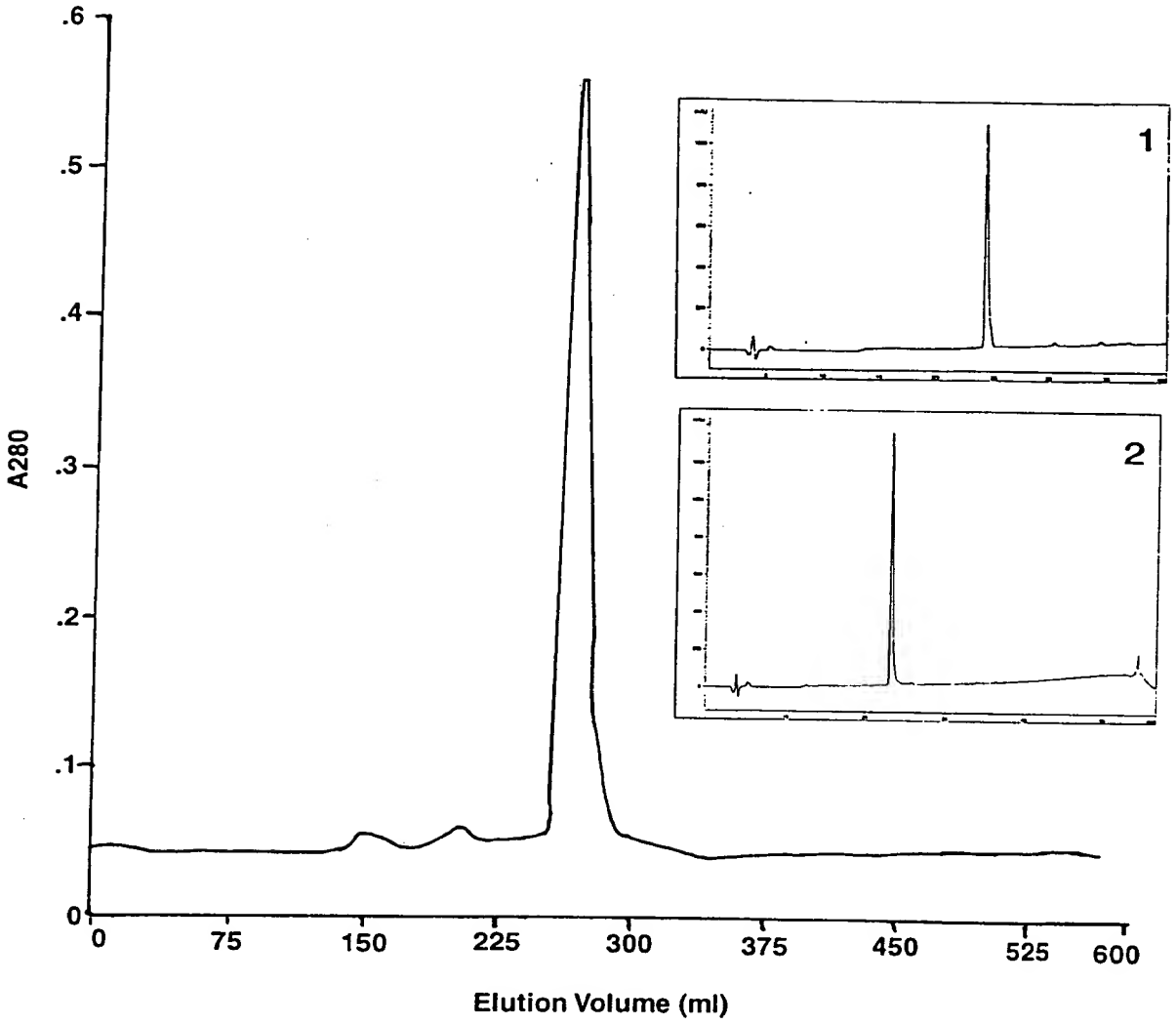
.6
.5
.4
.3
.2
.1
0
A280
0
75
150
225
300
375
450
525
600
Elution Volume (ml)
1
2

FIG. 2

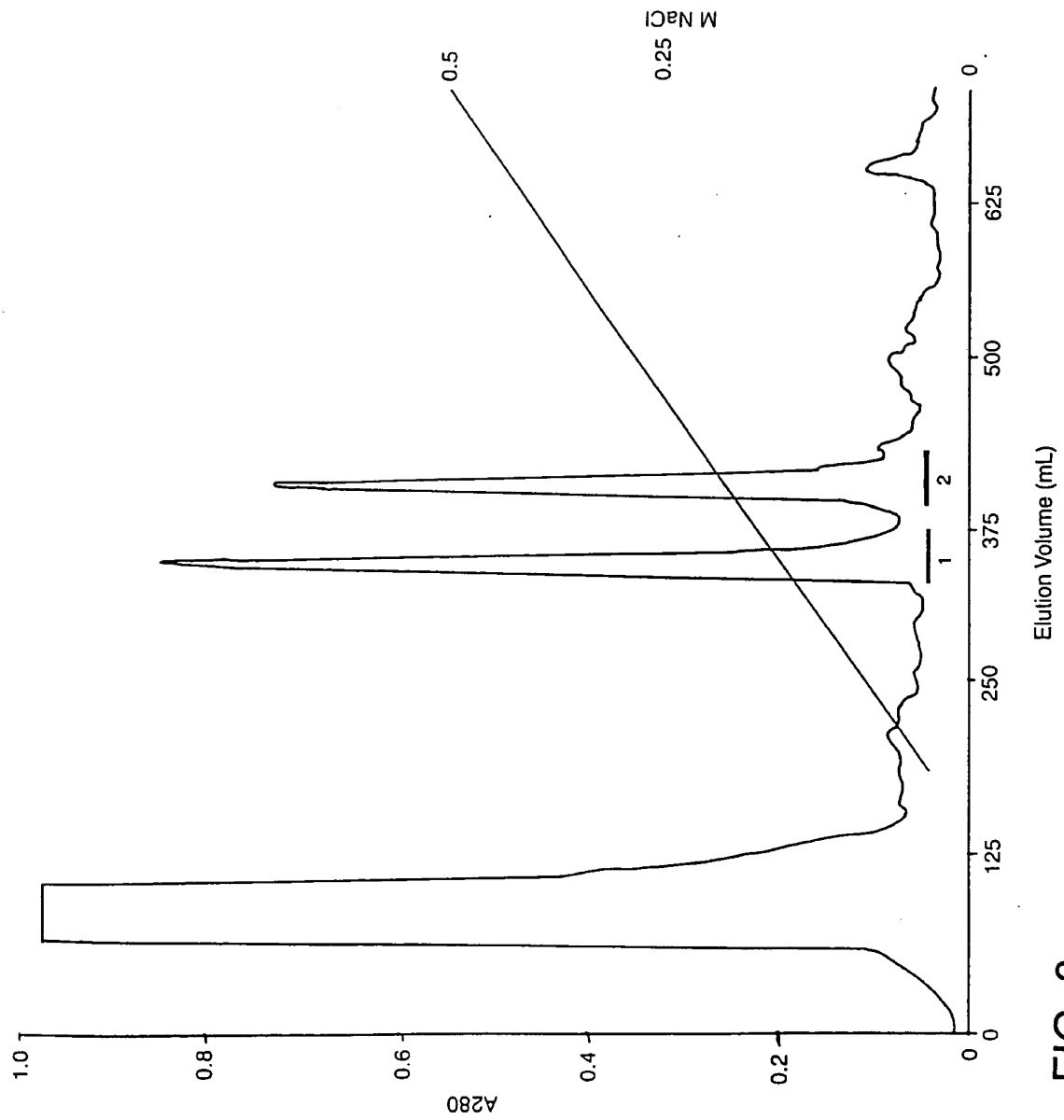
A280
1.0
0.8
0.6
0.4
0.2
0
0
125
250
375
500
625
Elution Volume (mL)
1
2
M NaCl
0.25
0.5
0

FIG. 3

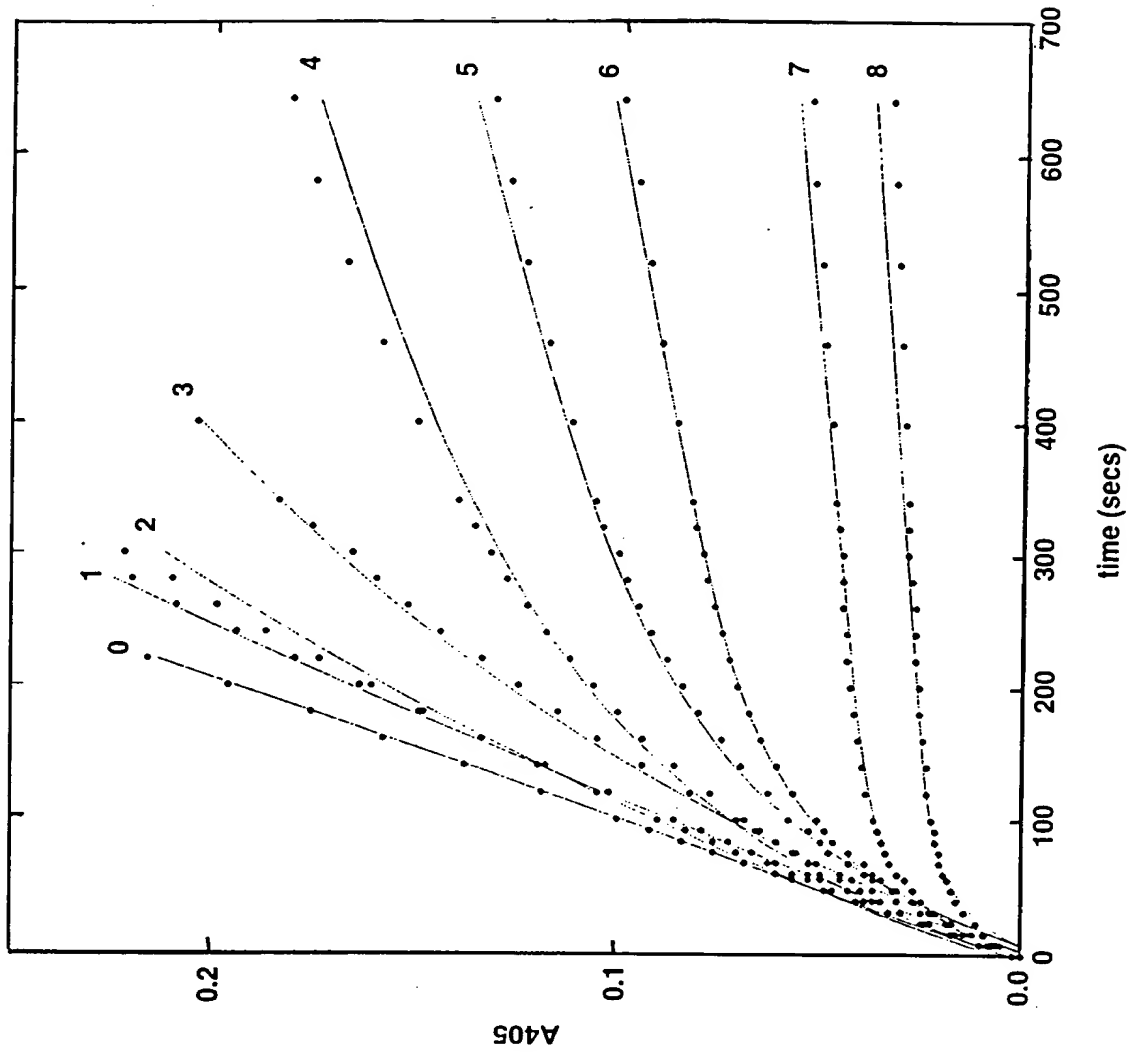

FIG. 4

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        10          20          30          40          50          59
KDRPDFCELP ADTGPCRVRF PSFYYNPDZK KCLZFIYGGC EGNANNFITK EECESTCGS        TXLN1
KDRPELCELP PDTGPCRVRF PSFYYNPDEQ KCLEFIYGGC EENANAFITK EECESTCGG        TXLN2
KDRPKFCHLP PKPGPCRAAI PRFYYNPHSK QCEKFIYGGC HGNANKFKTP DECNYTCLGVSL     TAC
  RPDFCLEP PYTGPCKARI IRYFYNAKAG LCQTFVYGGC RAKRNNFKSA EDCMRTCGGA       APRO
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FIG. 5

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ATG AAG GAC CGG CCT GAT TTT TGT GAA CTG CCT GCT GAC ACC GGA CCA TGT
 M   K   D   R   P   D   F   C   E   L   P   A   D   T   G   P   C

AGA GTC AGA TTC CCA TCC TTG TAC TAC AAC CCA GAT GAA AAA AAA TGC CTC
 R   V   R   F   P   S   F   Y   Y   N   P   D   E   K   K   C   L

GAG TTT ATT TAT GGT GGA TGC GAA GGG AAT GCT AAC GAT TTT ATG ACC AAA
 E   F   I   Y   G   G   C   E   G   N   A   N   N   F   I   T   K

GAG GAG TGT GAA AGC ACG TGT GG(N) AGT
 E   E   C   E   S   T   C   G     S
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FIG. 6

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ATG AAG GAC CGG CCT GAG TTG TGT GAA CTG CCT CCT GAC ACC GGA CCA TGT
 M   K   D   R   P   E   L   C   E   L   P   P   D   T   G   P   C

AGA GTC AGA TTC CCA TCC TTG TAC TAC AAC CCA GAT GAA CAA AAA TGC CTC
 R   V   R   F   P   S   F   Y   Y   N   P   D   E   Q   K   C   L

GAG TTT ATT TAT GGT GGA TGC GAA GAG AAT GAT AAC GCT TTT ATG ACC AAA
 E   F   I   Y   G   G   C   E   E   N   A   N   A   F   I   T   K

GAG GAG TGT GAA AGC ACG TGT CC(N) GGT
 E   E   C   E   S   T   C   G     G
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FIG. 7